Journal of Medical Virology 52:441–450 (1997)

Serological and Molecular Analysis of Hepatitis C Virus Envelope Regions 1 and 2 During Acute and Chronic Infections in Chimpanzees

Leen-Jan van Doorn,[1]* Kitty van Hoek,[1] Guy de Martinoff,[2] Fons Bosman,[2] Lieven Stuyver,[2] Ton Kos,[3] Inge Frantzen,[4] Peter Sillekens,[4] Geert Maertens,[2] and Wim Quint[1]

[1]*Delft Diagnostic Laboratory, Delft, The Netherlands*
[2]*Innogenetics NV, Gent, Belgium*
[3]*Biological Primate Research Center, Rijswijk, The Netherlands*
[4]*Organon Teknika BV, Boxtel, The Netherlands*

Acute and chronic Hepatitis C virus infections were investigated retrospectively in chimpanzees that had been infected from a single source. Anti-E1 and anti-E2 were detected in two of three chimpanzees with a chronic infection, but were first detected 1 to 2 years after inoculation. Sequence evolution of the E1 region in three animals over a period of 9 to 11 years revealed a mutation rate of 1.02 to 2.23 × 10^{-3} base substitutions per site per year. The acute phase viremia levels in acute infections which resolved appeared to be at least 10-fold higher than during the acute phase of chronic infections. During chronic infections, the viral load fell rapidly after the acute phase and remained at very low levels for several years. After 4 to 6 years, the viral load and liver enzymes increased again, suggesting reactivation of the infection. There was no clear temporal relationship between sequence evolution of the E1 region, changes in viral load, and the production of antibodies to the envelope proteins. ***J. Med. Virol. 52:441–450, 1997.***

KEY WORDS: quantitative HCV RNA; long-term follow-up; sequence evolution

INTRODUCTION

Infections with the Hepatitis C virus (HCV) are an important worldwide health problem. HCV is currently responsible for the majority of parenterally transmitted non-A, non-B hepatitis cases. The virus contains a positive-sense, single-stranded RNA genome of approximately 9,400 nucleotides with an open reading frame encoding about 3,010 amino acids. The genomic organization resembles that of other Flavivirus genera [Choo et al., 1991]. The genome appears to be highly heterogeneous, and at least 11 major types including numerous subtypes have been recognized [Maertens and Stuyver, 1996; Okamoto and Mishiro, 1994; Simmonds, 1995].

The chimpanzee is the only species other than man that is generally susceptible to infection by HCV. In previous studies we investigated retrospectively HCV infections in a cohort of chimpanzees which had been infected from a single source, containing genotype 1b virus [van Doorn et al., 1994]. Three chimpanzees developed a chronic disease with persistent viremia since 1983, whereas the remaining animals only experienced acute self-limiting disease with transient viremia. The hypervariable N-terminal region of the E2 region was investigated previously in detail. A close relationship between sequence evolution and specific humoral immune responses to linear epitopes has been observed, indicating a immune-selection mechanism of viral evolution [van Doorn et al., 1995].

In the present study, antibody production to recombinant E1 and E2, as well as sequence evolution of the E1 region during the course of acute and chronic infections were determined. Viremia levels were also measured and possible correlations with serological data were investigated.

MATERIALS AND METHODS

Chimpanzees (Pan troglodytes) Phil, Coen, Peggy, Hans, Socrates, Sophie, and Zeef were inoculated between 1982 and 1988 as described in detail previously [van Doorn et al., 1994; 1995]. The original inoculum was obtained from an acute phase plasma from a chim-

Contract grant sponsor: Dutch Government as part of Eureka project EU680 "Hepatitis C"; Contract grant number: EU68021; Contract grant sponsor: Flemish Government as part of the Eureka project EU680 "Hepatitis C"; Contract grant number: IWT 940072.

*Correspondence to: L.J. van Doorn, Delft Diagnostic Laboratory, R. de Graafweg 7, P.O. Box 5010, 2600 GA Delft, The Netherlands. E-mail: L.J.van.Doorn@ddl.nl

Accepted 10 April 1997

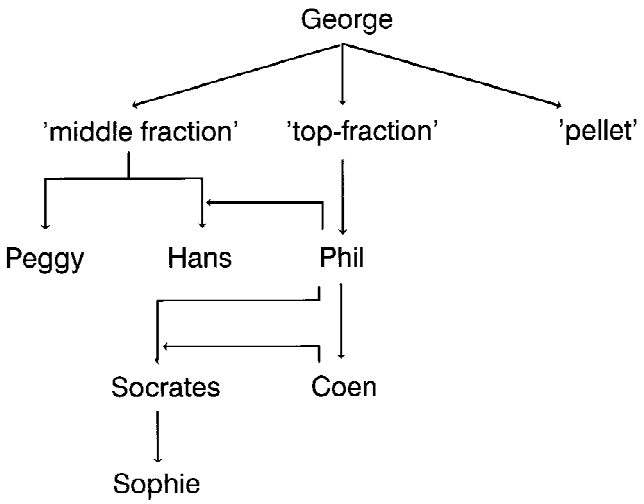

Fig. 1. Inoculation schedule of the chimpanzees, described in this study [see also van Doorn et al., 1994].

panzee that had been challenged with a contaminated Factor VIII preparation [Tsiquaye et al., 1980]. This acute phase plasma was ultracentrifuged several times, and two different fractions were used to inoculate further animals, as shown in Figure 1. Chimpanzee Phil received the so-called top-fraction in 1982, whereas chimpanzee Peggy was inoculated with material from the middle fraction in 1983.

In an attempt to neutralize viral infectivity, plasma obtained from Phil at 165 days after inoculation was mixed with a sample from the middle fraction and inoculated into chimpanzee Hans in 1983. Chimpanzee Coen, who had been inoculated previously with HIV 1, was inoculated in 1983 with plasma obtained from Phil, 35 days after inoculation. Plasma from Phil obtained 62 days after inoculation and plasma from Coen obtained at 192 days after inoculation were mixed, complexed with anti-IgG, and inoculated into chimpanzee Socrates in 1987. Forty ml of acute phase plasma was inoculated into chimpanzee Sophie in 1987. Finally, in 1988, chimpanzee Zeef received a mixture of plasma from Phil (obtained 1560 days after inoculation), Hans (obtained 1290 days after inoculation), Peggy (obtained 1380 days after inoculation), and one other chimpanzee that had been infected with the same inoculum as Phil (obtained 1380 days after inoculation). The aim of this experiment was to assess the presence of infectious virus in any of these animals long after inoculation.

All plasma samples were obtained by venipuncture and stored at −20°C in aliquots. The acute phase is defined as the period between first elevation of alanine aminotransferase (ALT) and/or gamma-glutamyl transferase (g-GT) until normalization of these liver-specific enzymes.

The HCV isolate used in this study has been classified as genotype 1b. Serial analysis of the presence of HCV RNA and antibodies to specific antigens has been described earlier [van Doorn et al., 1994]. HCV RNA remained detectable in chimpanzees Phil, Peggy, and Hans, indicating development of a chronic HCV infection.

In chimpanzees Socrates and Sophie, HCV RNA was detected from days 25 and 22, and it remained detectable for 80 and 49 days, respectively. In all samples obtained from these two animals after the acute phase, HCV RNA was never detected again, indicating an acute HCV infection which resolved.

Envelope Antibodies

Antibodies against E1 and E2 epitopes were measured by prototype versions of Innotest HCV E1Ab and Innotest HCV E2Ab (Innogenetics, Gent, Belgium). All envelope antigens were derived from a European subtype 1b isolate. Truncated E1 and E2 proteins were expressed by the vaccinia system and purified from mammalian cells transfected with recombinant vaccinia clones comprising amino acid residues 192 to 326 (E1) or 384-673 (E2). Both proteins lacked the membrane-anchor regions. Two peptides comprising the major subtype 1b linear epitopes were also employed; epitope A comprises aa 313-326, and epitope B comprises aa 192-226.

HCV-RNA PCR

HCV RNA was isolated from plasma by mixing 100μl with 400μl of lysis buffer [5M guanidinium thiocyanate, 0.125M Tris HCl, pH 7.4, 0.3M sodium acetate, freshly supplemented with 80μg/ml poly (A) (used as a carrier for precipitation) and 1.25% (v/v) 2-mercaptoethanol]. After vigorous mixing and incubation at 65°C for 10 minutes, the samples were cooled on ice and 500μl of cold isopropanol was added. Mixtures were centrifuged at 14.000 × g for 20 minutes at 4°C. Pellets were washed once with 500μl of cold 80% ethanol. The pellet was dissolved in 30μl of RNAse-free water. Ten μl of this solution was used immediately for cDNA synthesis, by adding the antisense cDNA primer (20 pmoles) and dNTPs (1mM final concentration), followed by denaturation for 2 minutes at 80°C and cooling on ice. Buffer (final concentrations 50 mM Tris-HCl, pH 8.0, 3 mM $MgCl_2$, 75 mM KCl, 0.01 M DTT), 200 units of M-MLV-reverse transcriptase (Gibco-BRL), and 30 units of RNasin (Promega) were added to a final volume of 25 μl. After incubation at 37°C for 60 minutes, the reverse transcriptase was inactivated at 95°C for 10 minutes and 75μl of PCR mix, containing the sense primer, 0.25 units of Taq DNA polymerase (SuperTaq, SphaeroQ, Leiden, The Netherlands), and the appropriate buffer (final concentrations: 10 mM Tris-HCl pH 9.0, 50 mM KCl and 2.5 mM $MgCl_2$) were added. The PCR program consisted of a pre-incubation at 95°C for 1 minute followed by 40 cycles of 1 minute at 95°C, 1 minute at 52°C, and 1 minute at 74°C. Nested PCR was carried out by transfer of 1μl of the first round PCR product into a new PCR reaction mixture, containing nested primers. PCR products were examined on 2% agarose gels.

For PCR aimed at the 5′ UTR, primers HCV19 and

TABLE I. Primer Sequences and Positions

Primer	Sequence (5'- to -3')[a]	Position[b], (polarity)
HCV19	GTGCACGGTCTACGAGACCT	−1 to −20, (−)
HCV35	TTGGCGGCCGCACTCCACCAT	
	GAATCACTCCCC	−319 to −297, (+)
NCR3	GGGGCGGCCGCCACCATRRA	
	TCACTCCCCTGTGAGG	−315 to −289, (+)
NCR4	CACTCTCGAGCACCCTATCAG	
	GCAGTACC	−66 to −47, (−)
HCV886S	CTGTCYTGYTTGACYRTCCCAGC	+544 to +566, (+)
HCV892S	GYTTGACYRTCCCAGCTTMCGCT	+551 to +573, (+)
HCV1147AS	TCAACGCCGGCAAAGAGTAGCATCAC	+1120 to +1146, (−)
HCV1150AS	CCCGTCAACGCCGGCAAAGAGTAGC	+1125 to +1150, (−)
HCV983	GG i GACCAGTTCATCATCAT	+963 to +983, (−)

[a]Y = C/T; R = A/G; M = A/C; i = inosine; underlined sequences are not HCV specific.
[b]Positions relative to the initiation codon of the polyprotein.

HCV35 were used in the first round, and primers NCR4 and NCR3 were used in the nested reaction.

The performance of this method was evaluated by assessment of the HCV proficiency panel as described previously [Zaaijer et al., 1993] and showed good sensitivity and specificity (data not shown).

For amplification of the E1 region, primers HCV1150AS and HCV886S were used in the first round, and HCV892S and HCV1147AS were used for nested PCR. Primer sequences are shown in Table I.

Cloning and Sequencing

PCR fragments comprising the E1 sequences were ligated into the pGEM-T vector (Promega) according to the manufacturer's instructions and transformed into *Escherichia coli* JM109, using standard procedures. Two primers, flanking the multiple cloning sites, as well as internal primer HCV983 were used for sequence analysis. Sequences of 546 bp were read manually and analysed by PC-gene software (Intelligenetics, Mountain View, CA). Phylogenetic analyses were performed by de PHYLIP package version 3.5c [Felsenstein, 1993].

Nucleic Acid Sequence Based Amplification (NASBA)

A quantitative NASBA assay [Vandamme et al., 1995; van Gemen et al., 1995a;b] (Organon Teknika, Boxtel, The Netherlands) was used to measure levels of HCV RNA in serum. Briefly, HCV RNA was isolated by the guanidinium thiocyanate/silica method [Boom et al., 1990] in the presence of three different internal control RNAs, each at a 1 log different concentration. Part of the 5′ UTR was amplified by NASBA [Sillekens et al., 1994] and the products were quantitated by electrochemiluminescence (ECL). HCV RNA concentrations were expressed as genome equivalents per ml.

Competitive RT-PCR

A PCR fragment, comprising nt −341 to +410 of the HCV RNA genome, obtained from a genotype 1b isolate, was cloned into pGEM-T (Promega). A 51-bp insert was introduced into the SphI site at position −63 in the 5′ UTR. RNA transcripts were synthesized from purified recombinant plasmid by the Riboprobe kit using T7 RNA polymerase (Promega) and serially diluted in DEPC-treated water, containing 1mg/ml poly (A) as a carrier. Ten-fold dilutions were tested by RT-PCR and the detection limit was reproducibly established at the 10^{-10} dilution.

For competitive PCR, 10-fold dilutions of the competitor RNA ranging from 10^{-5} to 10^{-10} were used. HCV RNA was isolated from 50μl of plasma, as described above, and dissolved in 50μl of RNAse-free water. Ten μl samples of this solution were used each with 2μl of a dilution of competitor RNA in standard cDNA-PCR reactions, as described above. For each series of samples, a known positive sample and a negative sample were tested in parallel. PCR fragments were visualized on a 2% agarose gel. Relative RNA quantities were assessed by visual examination.

RESULTS

Acute-resolved and chronic hepatitis C virus infections in chimpanzees were investigated retrospectively. Sequence evolution of the putative envelope 1 (E1) region was determined in chimpanzees Peggy, Hans, and Phil, which developed a chronic infection. HCV viremia levels were determined in serial samples both from acute-resolved and chronic infections. Antibodies to recombinant E1 and E2 proteins and peptides were measured.

Sequence Evolution of the E1 Region

Sequence evolution of the E1 region was determined by sequencing of independent clones of RT-PCR products, comprising nucleotides 574 to 1119 (amino acid residue 192 to 373) from various plasma samples. A total of 56 independent clones were sequenced and consensus nucleotide and amino acid sequences were determined for each sample.

For analysis of acute infections which resolved, clones were obtained from the first and the last HCV-RNA positive serum sample of the acute phase. In none of these animals was any sequence evolution observed during this brief period of viremia. The recombinant E1

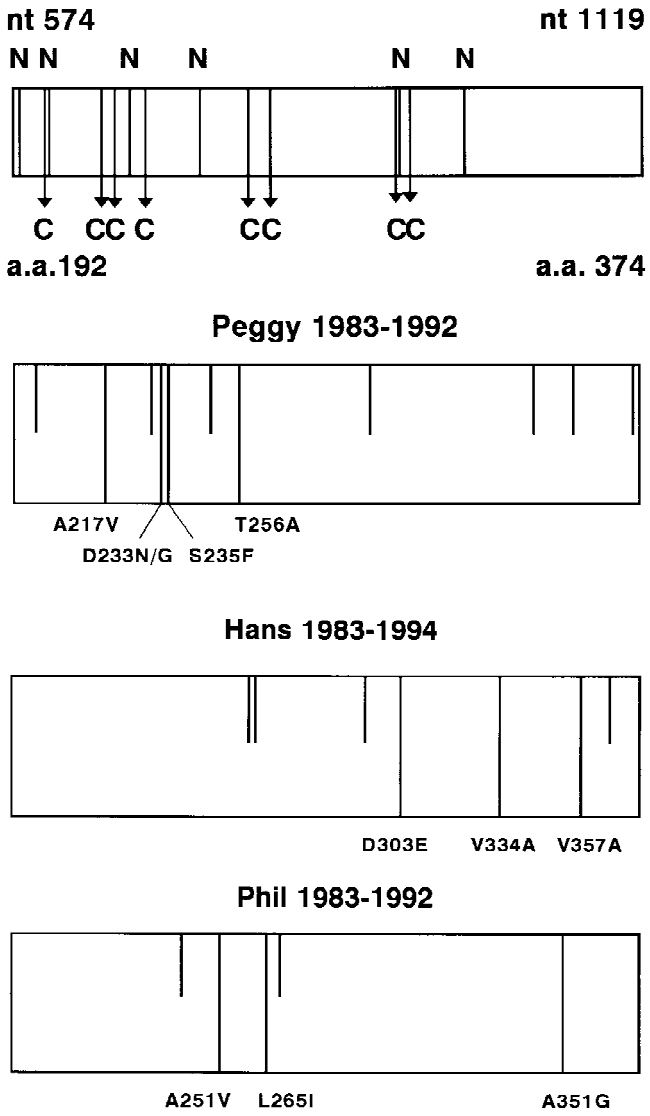

Fig. 2. Distribution of nucleotide substitutions with (long bars) or without (short bars) amino acid changes plotted along the E1 gene (amino acid residues 192 to 374) in 3 chronically infected chimpanzees. Amino acid changes are indicated below the long bars. N indicates potential N-linked glycosylation sites. C indicates location of cysteine residues.

protein differed at only 6 positions compared with the chimpanzee consensus sequences present during the acute phase (I192F, M199V, S209A, N232D, I312I, and T313S).

For analysis of the chronic infections in Phil, Peggy, and Hans, clones were obtained from the early acute phase as well as from samples obtained 9 to 11 years later. Figure 2 summarizes the sequence evolution in the 3 chronic infections. Three to 4 mutations causing amino acid changes occurred at different positions of the E1 region in the 3 chimpanzees. The 6 potential N-linked glycosylation sites as well as the 8 cysteine residues were completely conserved. Based on the consensus sequences, the mutation rate was estimated at between 1.02 and 2.23 $\times 10^{-3}$ base substitutions per site per year. All available E1 sequences were subjected to phylogenetic analysis and the resulting tree representing evolutionary distances is shown in Figure 3. Sequences obtained during the chronic phases from Peggy, Hans, and Phil form 3 separate clusters. Sequences obtained from all animals during the acute phase comprise a very homogeneous group. Molecular evolutionary distances are summarized in Table II.

Viremia Levels

Viremia levels obtained by quantitative NASBA measurements of HCV RNA from serial samples of chimpanzees are shown in Figure 4 (results from Hans were very similar to Peggy; results from Sophie were very similar to Socrates; these are therefore not shown).

In each animal, viremia preceeded the peak of serum ALT in the acute phase. Although viremia levels already decreased before the ALT peak, complete disappearance of HCV-RNA, as determined by PCR, in chimpanzees Socrates, Sophie, and Zeef coincided with ALT normalization. Remarkably, the peak levels of viremia during the acute phase in the animals with acute-resolved infections were at least tenfold higher than those observed during the acute phases in animals with chronic infections. In chimpanzees Phil, Hans, and Peggy, RNA levels remained very low for prolonged periods after the acute phase. HCV-RNA levels during the acute phase were very low in Phil. However, 4 to 6 years later, viral titers increased again to considerably higher levels in the 3 chronically infected animals, and g-GT levels also increased. In order to confirm these quantitative HCV RNA measurements by NASBA, a number of samples from Socrates, Sophie, Zeef, Hans, Phil, and Peggy were also tested by competitive RT-PCR, resulting in very similar patterns of viral load (data not shown).

Envelope Antibodies

Chimpanzees Sophie and Socrates, with acute-resolved infections, and chimpanzee Phil, with a chronic infection, did not show significant antibody responses to any of the recombinant E1 and E2 antigens or E1 peptides (Fig. 4A and C, bottom panel). Chimpanzees Peggy and Hans also did not produce detectable antibodies to the E1 peptides, representing linear epitopes (data not shown). However, these animals did show a humoral immune response to the recombinant E1 and E2 proteins (Fig. 4B, bottom panel). In both chimpanzees with chronic infections, antibodies to E1 and E2 appeared 1 to 2 years after primary infection, which is remarkably late.

DISCUSSION

There are four hierarchical levels of genetic heterogeneity of HCV, i.e. types, subtypes, isolates, and quasispecies, with decreasing levels of variability [Okamoto et al., 1994]. The different regions of the genome show different levels of sequence heterogeneity. The 5′UTR and core region are well conserved, whereas the

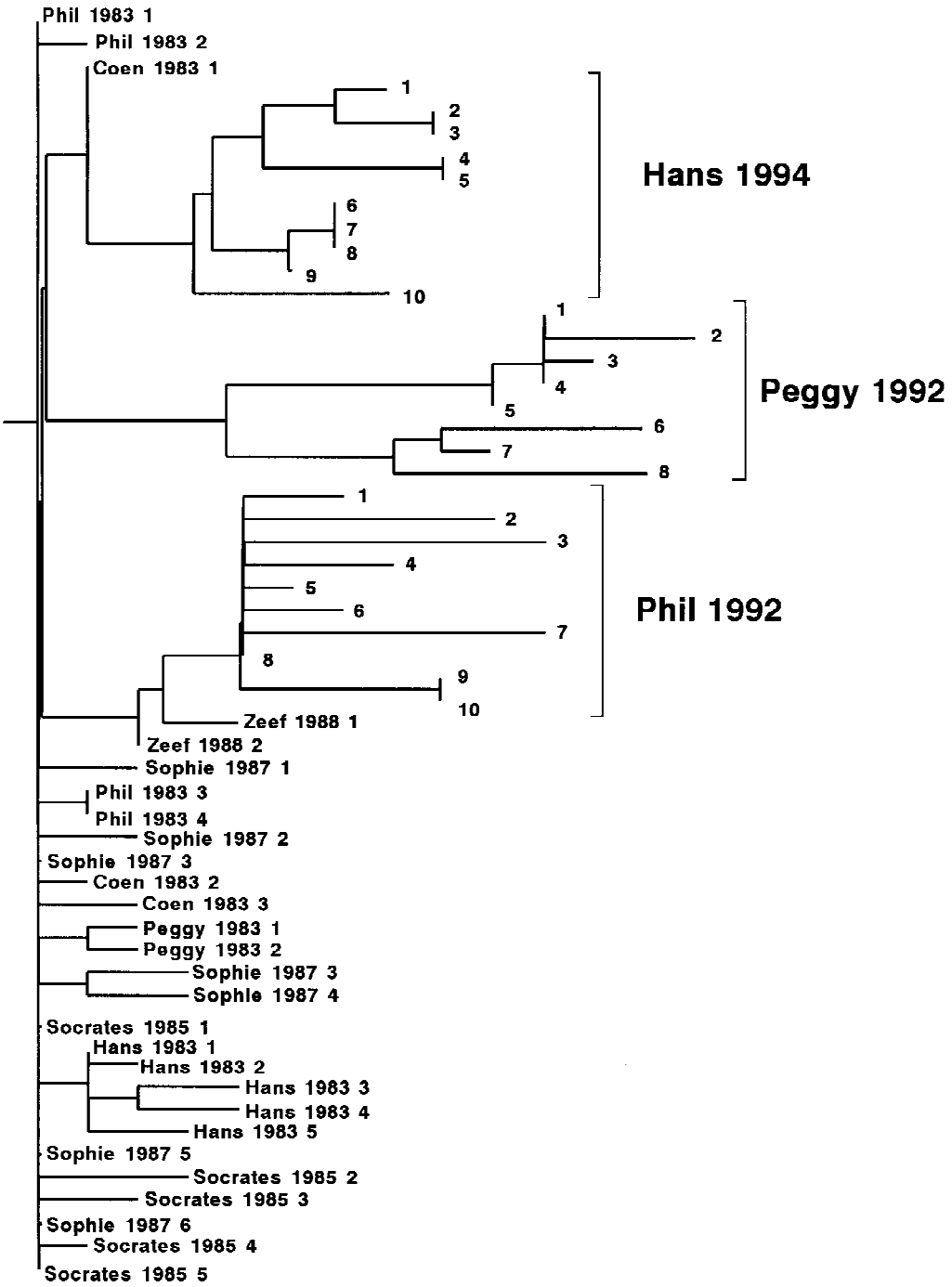

Fig. 3. Phylogenetic tree representing molecular evolutionary distances between 56 independent E1 sequences each comprising 546 nucleotides, obtained during acute and chronic phases from various chimpanzees. Sequences from Peggy, Phil, and Hans obtained after 9 to 11 years of chronic infection are indicated by numbers and are shown as separate clusters.

N-terminus of the putative envelope protein E2 is hypervariable and appears to be subject to immune selection, resulting in escape mutants [van Doorn et al., 1995; Kato et al., 1994; Sekiya et al., 1994; Weiner et al., 1992]. The apparent lack of protective immunity [Farci et al., 1992] may be attributed in part to sequence evolution of important immunological epitopes and therefore has important implications for the development of an effective vaccine.

In order to investigate the HCV envelope regions, the present study describes sequence evolution of the E1 region and the changes of the viral titer as well as measurement of antibodies to E1 and E2 epitopes in a group of chimpanzees which had previously been infected from a single source.

In order to establish whether changes in the E1 epitopes may be related to E1Ab production, sequence evolution of the E1 region was studied. Sequence analysis

TABLE II. Molecular Evolutionary Distances (± Standard Deviation) Among E1 Sequences From Chronic Phase Sera of Peggy, Phil, and Hans, and From Acute Phase Sera of All Animals

	Peggy 1992	Hans 1994	Phil 1992	Acute phase
Peggy 1992	0.016 ± 0.011			
Hans 1994	0.032 ± 0.004	0.009 ± 0.005		
Phil 1992	0.033 ± 0.005	0.026 ± 0.004	0.012 ± 0.005	
Acute phase	0.022 ± 0.004	0.015 ± 0.004	0.017 ± 0.004	0.005 ± 0.002

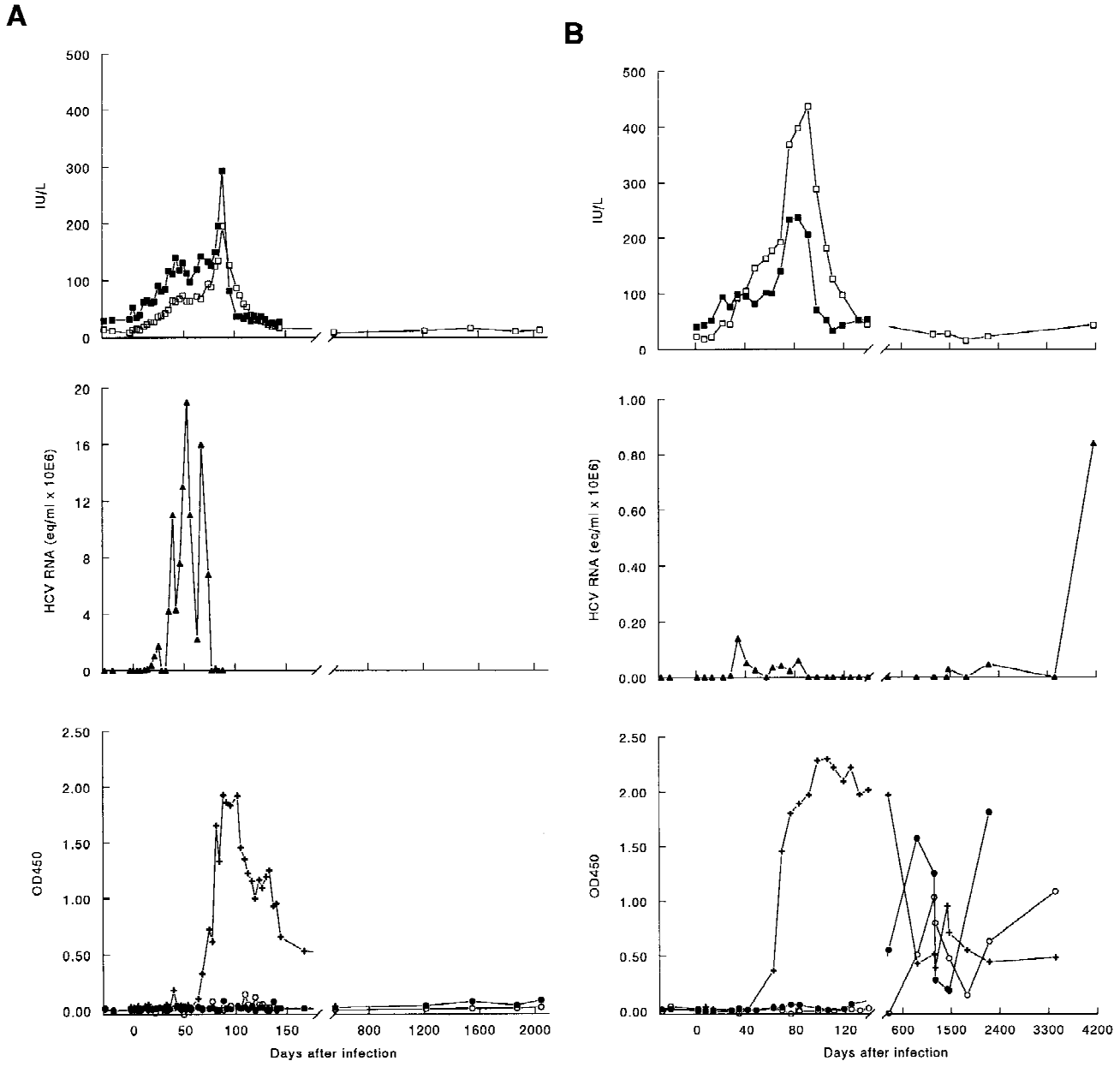

Fig. 4. Longitudinal analysis of HCV infection in chimpanzees Socrates **(A),** Peggy **(B),** and Phil **(C).** Upper panel shows levels of serum alanine aminotransferase (closed boxes) and gamma-transpeptidyl-transferase (open boxes). Middle panel shows HCV-RNA levels (closed triangles). Bottom panel shows antibody production to recombinant E1 (E1Ab; open circles), to recombinant E2 (E2Ab; closed circles), and to a peptide corresponding to the hypervariable region (anti-HVR; crosses). Scales for HCV-RNA genome equivalents as shown on the Y-axis are not identical in each figure. Results from Sophie were very similar to those from Socrates. Results from Hans were very similar to those of Peggy.

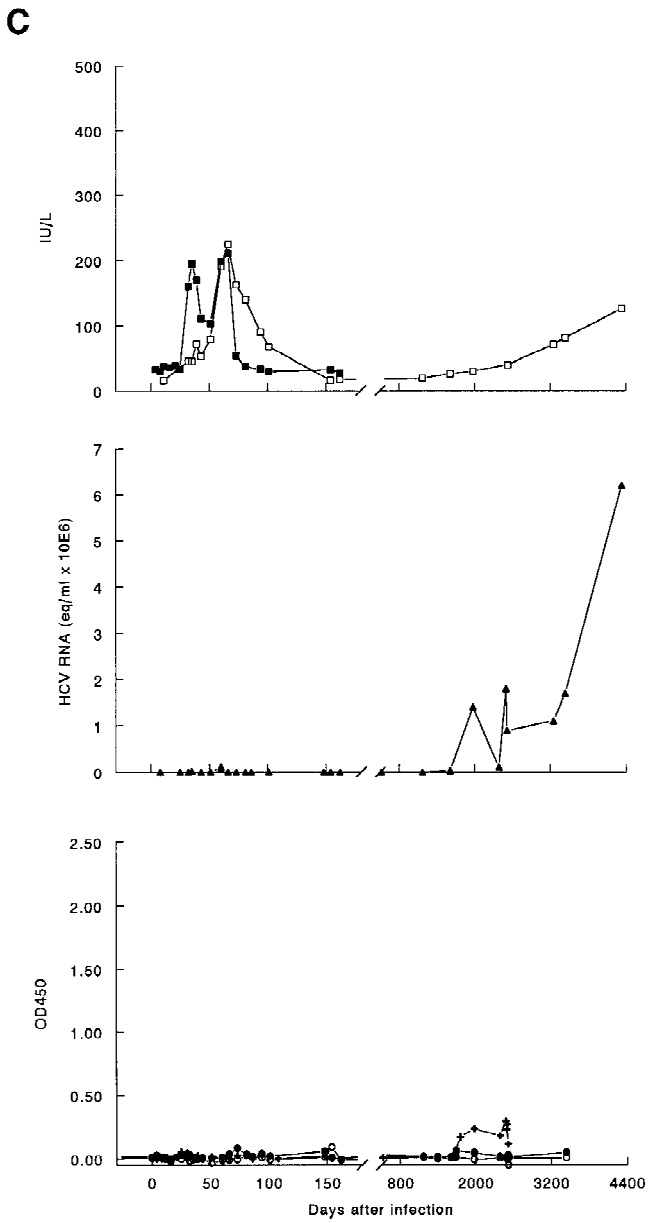

Fig. 4. Continued.

of a relatively limited number of variants results in only partial information of the entire population of closely related viral genomes (quasispecies) in a host. In the present study a total of 56 clones was sequenced.

Phylogenetic analysis of E1 sequences, as shown in Figure 3, indicates that all sequences obtained from acute-phase sera of all animals in this cohort indeed are highly similar, with average phylogenetic distances of only 0.005 ± 0.002. E1 sequences obtained 9 to 11 years after inoculation in Phil, Peggy, and Hans have clearly diverged from the acute phase variants, and each animal shows sequence evolution into a unique direction. These findings suggest that quasispecies evolution in chronic HCV infection is mainly driven by the host, and further indicate an immune-mediated influence on the quasispecies distribution of HCV [Maekewa et al., 1995]. In chimpanzee Peggy, the 2 main branches in the phylogenetic tree comprising the 1992 sequences suggest the emergence of 2 predominant variants. The evolution rate of the E1 sequence was comparable to other reports [Ogata et al., 1991; Okamoto et al., 1992; Yamaguchi et al., 1994].

In 1988, chimpanzee Zeef was inoculated with a mixture of sera from 4 other chimpanzees, including a sample obtained from Phil in 1987 (5 years after inoculation). The E1 sequence of Zeef reveals amino acid mutations A230V and A330G, which are identical to the mutations found in Phil in the 1992 sample. This is also shown by the intermediate position of the Zeef sequences in the phylogenetic tree and strongly suggests that Zeef indeed was infected by virus derived from Phil.

Phylogenetic distances among E1 sequences obtained from Phil (0.011 ± 0.005), Peggy (0.016 ± 0.011), and Hans (0.009 ± 0.005) during the chronic phase indicate an increase of the phylogenetic distances as compared to the clones obtained during the acute phases (0.005 ± 0.002). This might reflect an increasing divergence among the quasispecies during long-term HCV infections. However, validation of this hypothesis would require analysis of a much larger number of independent clones. The phylogenetic distances between E1 sequences from the chronic phase of chimpanzees Phil, Hans, and Peggy varied between 0.026 and 0.033. These distances are still lower than the mean distance (0.097 ± 0.029) between E1 sequences of independent subtype 1b isolates (Stuyver et al., 1994).

The amino acid sequence of the recombinant E1 protein was very similar to the E1 sequence deduced from the infected animals. Moreover, antibody responses to the recombinant E1 protein are frequently observed in infected humans, which are all infected with distinct virus variants and genotypes. Therefore it is very unlikely that this accounted for the lack of anti-E1 detection during the first stage of the infection.

The level of envelope antibody production may also be influenced by the viremia level. There appears to be a correlation between the level of anti-E1 antibodies and the long-term response to interferon-alpha treatment, reflecting a decrease of the level of viremia [Maertens et al., 1994, 1995]. In order to investigate this issue, HCV RNA levels were monitored in serial samples from several chimpanzees by quantitative NASBA and competitive RT-PCR. The NASBA quantitation method appears to be highly reproducible [van Gemen et al., 1995a]. In order to confirm the relative changes in HCV RNA levels over time, quantitation by competitive RT-PCR using tenfold dilutions was also employed. Although visual inspection of agarose gels does not allow accurate measurement of the precise number of HCV RNA molecules, it permits correct assessment of the relative changes in viral load in serial samples. During the acute phase of disease, acute viremia preceded the ALT peak, which is in accordance with previous observations [Farci et al., 1992; Beach et

al., 1992; Shimizu et al., 1990]. It is remarkable that the peak level of viremia in the two acute-resolved infections (Sophie and Socrates, Fig. 4A, middle panel) appeared to be at least tenfold higher than in the three chronic infections (Phil, Peggy, and Hans, Fig. 4B, C, middle panel). This phenomenon may be of crucial importance in the difference between acute resolved and chronic infection. Hypothetically, during the acute phase of the infection, higher levels of viremia may offer a powerful stimulus to the immune system resulting in an effective immune response, whereas lower viremia levels may evoke only a weak immune response.

Another interesting observation is the course of viremia in chronic infections. After the acute phase, HCV RNA remained present at very low levels. However, several years later, the viral titer was raised again. During prolonged chronic infection a correlation between increasing HCV RNA concentrations and the duration of chronic infection has been indicated [Kato et al., 1993]. Moreover, high-titer viremia appears to correlate with advanced stages of disease in patients [Gretch et al., 1994; Hagiwara et al., 1993]. This could also be consistent with the observed increase of gamma-GT levels in the 3 chronically infected chimpanzees Peggy, Hans, and Phil, suggesting reactivation of the viral replication over time. The liver-specific enzyme levels in the acute-resolved cases (Sophie and Socrates) were never elevated during follow-up.

The E1 protein contains epitopes that elicit efficient humoral immune responses in humans [Maertens et al., 1994; Koziel et al., 1992; Sällberg et al., 1993]. However, antibody production against envelope proteins appeared to be relatively inefficient in chimpanzees. This is consistent with the results from the first attempts to vaccinate chimpanzees with recombinant envelope proteins. Administration of recombinant envelope proteins only elicited weak and transient humoral immune responses which resulted in protection to challenges with low doses of the homologous virus in 5 of 7 vaccinees with the highest anti-E1/E2 titers [Choo et al., 1994].

Antibodies to the recombinant E1 protein were first detected in Peggy and Hans during the early chronic stage, 1 to 2 years after primary infection, whereas no antibodies to linear peptides derived from the E1 region could be detected. These results indicate that humoral immune responses to envelope epitopes may take prolonged periods after infection. Since the divergence of E1 in Phil was comparable to that in the other chronically infected chimpanzees, although there was no detectable antibody, humoral responses are not the source of E1 variation.

The situation for the E2 protein is different. Specific antibodies to corresponding peptides from the N-terminus of E2 have been detected early during the acute phase of disease, whereas no antibody response could be measured to the complete recombinant E2 in the same sera. This can be explained by the fact that 13 of the 28 amino acids comprising the hypervariable region (HVR) sequence differed between the recombinant E2 protein and the HVR of the chimpanzee inoculum. During the acute phase of the infection, high levels of specific antibodies to HVR were produced in all animals except Phil and remained detectable in Peggy and Hans [van Doorn et al., 1995]. These epitopes were presented as linear peptides exactly corresponding to the HVR sequence of the consensus sequence of the chimpanzee inoculum. Apparently, antibody production to more common epitopes of E2, which can be usually observed early in humans, is delayed in chimpanzees.

Chimpanzee Phil not only showed a very delayed response to HVR epitopes, but also did not produce any detectable antibodies to recombinant E1 and E2 proteins. Reactivity to core epitopes, as measured with the Line Immuno Assay (measuring antibodies to specific HCV proteins that are presented as immobilized antigens on a nitrocellulose strip; van Doorn et al., 1994) was first detected several years after inoculation. Conclusively, the overall immune reactivity to HCV epitopes seems to be particularly delayed in this animal.

These results might indicate that the chimpanzee is not an optimal model to study the immunological responses to HCV envelope proteins. However, the use of well-defined antigens with the proper conformation and posttranslational modifications in immunoassays is extremely important.

The recombinant E1 and E2 proteins used in this study were recognized by 86% and 91%, respectively, of several hundreds of human sera from blood donors and HCV-infected patients [Maertens; unpublished observations]. Antibodies to both linear and conformation-dependent epitopes of envelope proteins coexist with viremia in chronic HCV infection [Chien et al., 1993]. Recent studies have indicated E2Ab to be produced early after infection [Zaaijer et al., 1994].

Results from the present study do not indicate simple relationships between antigenic variation of the envelope proteins, antibody production, and levels of viremia. The quasispecies nature of the hypervariable region in E2 indicates the importance of mutants escaping the immune selection mechanism, driven by specific antibodies to linear epitopes from the HVR. It has been shown that antibody-mediated neutralization may prevent infectivity in chimpanzees [Farci et al., 1994] and can block viral attachment in vitro [Zibert et al., 1995]. Cellular immunity, especially to core and NS3, may be of major importance to discriminate between self-limited and chronic HCV infections [Diepolder et al., 1995]. Recently, escape mutants to a NS3-specific cytotoxic T-lymphocyte have been described in an infected chimpanzee [Weiner et al., 1995]. On the other hand, the possibility of multiple HCV infections with different or homologous inocula, the absence of cross-neutralising antibodies, and the apparent lack of long-lasting protective immunity indicate the absence of adequate immune responses in vivo [Farci et al., 1992].

The present study shows that chronic HCV infection is a highly dynamic process. Despite the absence of

simple relationships between evolution of the quasispecies distribution, the viremia levels, and the production of envelope antibodies, there seem to be several consistent features of acute-resolving and chronic HCV infections, e.g., the consistently higher viremia levels during the acute phase of acute-resolved infections, the long-term exacerbation of disease during chronic infection, and the host-dependent sequence evolution of the HCV quasispecies. The long-term follow-up suggested reactivation of the virus, leading to increased levels of HCV-RNA, production of antibodies to envelope proteins, and rise of liver-specific enzymes.

These results also indicate that the development of an effective vaccine against HCV may be difficult and will require future studies on the precise immunological mechanisms that play a role during HCV infection.

ACKNOWLEDGMENTS

We thank Ann Venneman, Paul Vandeponseele, and Annemie Ducatteeuw for their technical assistance, and Anton Plaisier for his assistance in preparation of the figures.

This study was conducted according to the ethical standards approved by the local Institutional Committee.

REFERENCES

Beach MJ, Meeks EL, Mimms LT, Vallari D, DuCharme L, Spelbring J, Taskar S, Schleicher JB, Krawczynski K, Bradley DW (1992): Temporal relationships of hepatitis C virus RNA and antibody responses following experimental infection of chimpanzees. Journal of Medical Virology 36:226–237.

Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME, van der Noordaa J (1990): Rapid and simple method for purification of nucleic acids. Journal of Clinical Microbiology 28: 495–503.

Chien DY, Choo QL, Ralston R, Spaete R, Tong M, Houghton M (1993): Persistence of HCV despite antibodies to both putative envelope glycoproteins. Lancet 342:933.

Choo Q-L, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby A, Barr PJ, Weiner AJ, Bradley DW, Kuo G, Houghton M (1991): Genetic organization and diversity of the hepatitis C virus. Proceedings of the National Academy of Sciences USA 99:2451–2455.

Choo Q-L, Kuo G, Ralston R, Weiner A, Chien D, Van Nest G, Han J, Berger K, Thudium K, Kuo C, Kansopon J, McFarland J, Tabrizi A, Ching K, Moss B, Cummins LB, Houghton M, Muchmore E (1994): Vaccination of chimpanzees against infection by the hepatitis C virus. Proceedings of the National Academy of Sciences USA 91:1294–1298.

Diepolder H, Zachoval R, Hoffman RM, Wierenga EA, Santantonio T, Jung MC, Eichenlaub D, Pape GR (1995): Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. Lancet 346: 1006–1007.

Farci P, Alter HJ, Govindarajan S, Wong DC, Engle R, Lesniewski RR, Mushahwar IK, Desai SM, Miller RH, Ogata N, Purcell RH (1992): Lack of protective immunity against reinfection with hepatitis C virus. Science 258:135–140.

Farci P, Alter HJ, Wong DC, Miller RH, Govindarajan S, Engle R, Shapiro M, Purcell RH (1994): Prevention of hepatitis C virus infection in chimpanzees after antibody-mediated in vitro neutralization. Proceedings of the National Academy of Sciences USA 19:7792–7796.

Felstenstein J (1993): PHYLIP: Phylogeny Inference Package, version 3.5c. Seattle, WA: Univ. of Washington.

Gretch D, Corey L, Wilson J, dela Rosa C, Willson R, Carithers R, Busch M, Hart J, Sayers M, Han J (1994): Assessment of hepatitis C virus RNA levels by quantitative competitive RNA polymerase chain reaction: High-titer viremia correlates with advanced stage of disease. Journal of Infectious Diseases 169:1219–1225.

Hagiwara H, Hayashi N, Mita E, Naito M, Kasahara A, Fusamoto H, Kamada T (1993): Quantitation of hepatitis C virus RNA in serum of asymptomatic blood donors and patients with type C chronic liver disease. Hepatology 17:545–550.

Kato N, Yokosuka O, Hosoda K, Ito Y, Ohto M, Omata M (1993): Quantification of Hepatitis C virus by competitive reverse transcription-polymerase chain reaction: increase of the virus in advanced liver disease. Hepatology 18(1):16–20.

Kato N, Ootsuyama Y, Sekiya H, Ohkoshi S, Nakazawa T, Hijikata M, Shimotohno K (1994): Genetic drift in hypervariable region 1 of the viral genome in persistent hepatitis C virus infection. Journal of Virology 68(8):4776–4784.

Koziel MJ, Dudley D, Wong JT, Dienstag J, Houghton M, Ralston R, Walker BD (1992): Intrahepatic cytotoxic T lymphocytes specific for hepatitis C virus in persons with chronic hepatitis. Journal of Immunology 149:3339–3344.

Maekewa S, Enomoto N, Kurosaki M, Marumo F, Sato C (1995): Host dependent variation of hepatitis C virus phylogenetic analyses. Archives of Virology 140:2123–2133.

Maertens G, Stuyver L (1996): Genotypes and genetic variation of hepatitis C virus. In Zuckerman A, Harrison T (eds): "Molecular Medicine of Hepatitis Molecular Medical Science series." Chichester: John Wiley & Sons, pp 183–234.

Maertens G, Ducatteeuw A, Stuyver L, Vandeponseele P, Venneman A, Wyseur A, Bosman F, Heijtink RA, de Martinoff G (1994): Low prevalence of anti-E1 antibodies reactive to recombinant type 1b E1 envelop protein in type 2, 3, and 4 sera, but high prevalence in subtypes 1a and 1b. In Nishioka K, Suzuki H, Mishiro S, Oda T (eds): "Viral Hepatitis and Liver Disease." Tokyo: Springer Verlag Tokyo, pp 314–316.

Maertens G, Ducatteeuw A, Barlet V, Zarksi JP, de Martinoff G, Bosman F, Stuyver L, Brouwer JT, Heijtink RA (1995): E1 Antibody level monitoring predicts long-term resolution of hepatitis C virus infection. Hepatology 22:355A.

Ogata N, Alter HJ, Miller RH, Purcell RH (1991): Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. Proceedings of the National Academy of Sciences USA 88:3392–3396.

Okamoto H, Mishiro S (1994): Genetic heterogeneity of hepatitis C virus. Intervirology 37:68–76.

Okamoto H, Kojima M, Okada S-I, Yoshizawa H, Iizuka H, Tanaka T, Muchmore E, Peterson DA, Mishiro S (1992): Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: Variability and stability. Virology 190:894–899.

Sällberg M, Ruden U, Wahren B, Magnius LO (1993): Antigenic region within the hepatitis C virus envelope 1 and non-structural proteins: identification of an IgG3-restricted recognition site within the envelope 1 protein. Clinical and Experimental Immunology 91:489–494.

Sekiya H, Kato N, Ootsuyama Y, Nakazawa T, Yamaguchi K, Shimotohno K (1994): Genetic alteration of the putative envelope proteins encoding region of the hepatitis C virus in the progression to relapsed phase from acute hepatitis: humoral immune response to hypervariable region 1. International Journal of Cancer 57:664–670.

Shimizu YK, Weiner AJ, Rosenblatt J, Wong DC, Shapiro M, Popkin T, Houghton M, Alter HJ, Purcell RH (1990): Early events in hepatitis C virus infection of chimpanzees. Proceedings of the National Academy of Sciences USA 87:6441–6444.

Sillekens P, Kok W, van Gemen B, Lens P, Huisman H, Cuypers Th, Kievits T (1994): Specific detection of HCV RNA using NASBA as a diagnostic tool. In "Hepatitis C Virus GEMHEP." Paris: John Libbey Eurotext, pp 71–82. (ed. Groupe Francais d'Etudes Moleculaire des Hepatitis)

Simmonds P (1995): Variability of Hepatitis C virus. Hepatology 21: 570–583.

Stuyver L, van Arnhem W, Wyseur A, Hernandez F, Delaporte E, Maertens G (1994): Classification of hepatitis C viruses based on phylogenetic analysis of the envelope 1 and nonstructural 5B regions and identification of five additional subtypes. Proceedings of the National Academy of Sciences USA 91:10134–10138.

Tsiquaye KN, Bird RG, Tovey G, Wyke RJ, Williams R, Zuckerman AJ (1980): Further evidence of cellular changes associated with non-A, non-B hepatitis. Journal of Medical Virology 5:63–71.

Vandamme A-M, Van Dooren S, Kok W, Goubau P, Fransen K, Kievits T, Schmit J-C, De Clercq E, Desmeyter J (1995): Detection of HIV-1 RNA in plasma and serum samples using the NASBA amplification system compared to RNA-PCR. Journal of Virological Methods 52:121–132.

Van Doorn LJ, Quint W, Tsiquaye K, Voermans J, Paelinck D, Kos T, Maertens G, Schellekens H, Murray K (1994): Longitudinal analysis of hepatitis C virus infection and genetic drift of the hypervariable region. Journal of Infectious Diseases 169:1226–1235.

Van Doorn LJ, Capriles I, Maertens G, DeLeys R, Murray K, Kos T, Schellekens H, Quint WGV (1995): Sequence evolution of the hypervariable region in the putative envelope region E2/NS1 of hepatitis C virus is correlated with specific humoral immune responses. Journal of Virology 69(2):773–778.

Van Gemen B, Kievits T, Romano J (1995a): Transcription based nucleic acid amplification methods like NASBA and 3SR applied to viral diagnosis. Reviews in Medical Virology 5:205–211.

Van Gemen B, van der Wiel P, van Beuningen R, Sillekens P, Jurriaans S, Dries C, Schoones R, Kievits T (1995b): The one-tube quantitative HIV-1 RNA NASBA: precision, accuracy, and application. PCR Methods and Applications 4:S177–S184.

Weiner AJ, Geysen HM, Christopherson C, Hall JE, Mason TJ, Saracco G, Bonino F, Crawford K, Marion CD, Crawford KA, Brunetto M, Barr PJ, Miyamura T, McHutchinson J, Houghton M (1992): Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: Potential role in chronic HCV infections. Proceedings of the National Academy of Sciences USA 89:3468–3472.

Weiner AJ, Erickson AL, Kansopon J, Crawford K, Muchmore E, Hughes AL, Houghton M, Walker CM (1995): Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T lymphocyte escape variant. Proceedings of the National Academy of Sciences USA 92:2755–2759.

Yamaguchi K, Tanaka E, Higashi K, Kiyosawa K, Matsumoto A, Furuta S, Hasegawa A, Tanaka S, Kohara M (1994): Adaptation of hepatitis C virus for persistent infection in patients with acute hepatitis. Gastroenterology 106:1344–1348.

Zaaijer HL, Cuypers HThM, Reesink HW, Winkel IN, Gerken G, Lelie PN (1993): Reliability of polymerase chain reaction for detection of hepatitis C virus. Lancet 341:722–724.

Zaaijer HL, Vallari DS, Cunningham M, Lesniewski R, Reesink HW, van der Poel CL, Lelie PN (1994): E2 and NS5: new antigens for detection of hepatitis C virus antibodies. Journal of Medical Virology 44:395–397.

Zibert A, Schreier E, Roggendorf M (1995): Antibodies in human sera specific to hypervariable region 1 of hepatitis C virus can block viral attachment. Virology 208:653–661.